

(19)



Europäisches Patentamt

European Patent Office

Office européen des brevets



(11)

EP 1 016 710 A2

(12)

## EUROPEAN PATENT APPLICATION

(43) Date of publication:  
05.07.2000 Bulletin 2000/27

(51) Int. Cl.<sup>7</sup>: C12N 1/20, C12N 15/11,  
C07K 14/245

(21) Application number: 99125263.6

(22) Date of filing: 17.12.1999

(84) Designated Contracting States:  
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU  
MC NL PT SE  
Designated Extension States:  
AL LT LV MK RO SI

(30) Priority: 30.12.1998 RU 98124016  
09.03.1999 RU 99104431

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Remarks:

The biological material has been deposited with  
VKPM under numbers B-7728 / B-7729 / B-7731  
/ B-7730 / B-7719 / B-7722 / B-7707 / B-7712 / B-  
7708 / B-7753 / B-7754 / B-7752 / B-7713 / B-7714 /  
B7748 / B-7715 / B-7716 / B-7718.

## (54) Method for producing L-amino acids

(57) A bacterium belonging to the genus *Escherichia* and having an ability to produce an L-amino acid, wherein the ability to produce the L-amino acid is increased by increasing an expression amount of an L-amino acid excretion protein, and a method for producing the L-amino acid using the bacterium.

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## Description

## Technical Field

5 [0001] The present invention relates to a method for producing an amino acid. In particular, the present invention relates to an L-amino acid-producing bacterium belonging to the genus *Escherichia* and a method for producing L-amino acids, more specifically, L-glutamic acid, L-lysine, L-threonine, L-alanine, L-histidine, L-proline, L-arginine, L-valine, and L-isoleucine, using the bacterium.

## 10 Background Art

[0002] For production of an L-amino acid by fermentation, a strain isolated from the natural world or an artificial mutant of the strain has been used to improve productivity. For example, in the case of L-lysine, many artificial mutants producing L-lysine are known, and most of them are mutants resistant to S-2-aminoethylcysteine (AEC) and belong to the genus *Brevibacterium*, *Corynebacterium*, *Bacillus* or *Escherichia*. Also, there have been proposed various technics for increasing amino acid production such as use of a transformant obtained by using a recombinant DNA (U.S. Patent No. 4,278,765).

[0003] The technics are mostly based on enhancement of an activity of an enzyme involved in an amino acid biosynthetic pathway, conversion of the enzyme to that desensitized in inhibition and the like (As to bacterium belonging to the genus *Escherichia*, see Japanese Patent Application Laid-Open No. 56-18596 (1981) and International Publication No. WO 95/16042).

[0004] On the other hand, as an example of improvement of amino acid productivity by enhancing an amino acid excretion protein, a bacterium belonging to the genus *Corynebacterium* in which an L-lysine excretion gene, *lysE* is enhanced is known. However, as to bacteria belonging to the genus *Escherichia*, it is unknown even whether an L-amino acid excretion protein is present or not. Therefore, it is unknown whether enhancement of the L-amino acid excretion protein is effective in L-amino acid production using a bacterium belonging to the genus *Escherichia* or not.

[0005] Although the entire nucleotide sequence of *E. coli* strain K-12 belonging to the genus *Escherichia* has been already determined (Science, 277, 1453-1474(1997)), there are a large number of proteins of which functions are unknown.

## 30 Disclosure of the Invention

[0006] An object of the present invention is to obtain a protein participating in excretion of an L-amino acid, thereby providing a strain improved in L-amino acid productivity and an improved method for producing an L-amino acid by fermentation.

[0007] The inventors have conducted screening for the protein participating in excretion of an L-amino acid. As a result, the present inventors have found that a yield of an L-amino acid based on consumed sugar is increased when a particular gene is enhanced. On the basis of the finding, the present invention has been completed.

[0008] Thus, the present invention provides a bacterium belonging to the genus *Escherichia* and having an ability to produce an L-amino acid, wherein the ability to produce the L-amino acid is increased by increasing an expression amount of at least one protein selected from the group consisting of the following proteins of (A) to (H) (hereinafter also referred to as "the bacterium of the present invention"):

- (A) a protein having an amino acid sequence shown in SEQ ID NO: 10 in Sequence Listing;
- 45 (B) a protein which has an amino acid sequence including deletion, substitution, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO: 10 in Sequence Listing, and which has an activity of increasing the ability to produce the L-amino acid of the bacterium having the protein;
- (C) a protein having an amino acid sequence shown in SEQ ID NO: 12 in Sequence Listing;
- (D) a protein which has an amino acid sequence including deletion, substitution, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO: 12 in Sequence Listing, and which has an activity of increasing the ability to produce the L-amino acid of the bacterium having the protein;
- 50 (E) a protein having an amino acid sequence shown in SEQ ID NO: 14 in Sequence Listing;
- (F) a protein which has an amino acid sequence including deletion, substitution, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO: 14 in Sequence Listing, and which has an activity of increasing the ability to produce the L-amino acid of the bacterium having the protein;
- 55 (G) a protein having an amino acid sequence shown in SEQ ID NO: 16 in Sequence Listing; or
- (H) a protein which has an amino acid sequence including deletion, substitution, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO: 16 in Sequence Listing, and which

has an activity of increasing the ability to produce the L-amino acid of the bacterium having the protein.

[0009] The bacterium of the present invention preferably an L-lysine-producing bacterium in which an expression amount of at least one protein selected from the group consisting of the proteins (A) to (D), (G) and (H) is increased; an L-glutamic acid-producing bacterium in which an expression amount of at least one protein selected from the group consisting of the proteins (A) to (H) is increased; an L-alanine-producing bacterium in which an expression amount of at least one protein selected from the group consisting of the proteins (C) and (D) is increased; an L-valine-producing bacterium in which an expression amount of at least one protein selected from the group consisting of the proteins (C) and (D) is increased; an L-histidine-producing bacterium in which an expression amount of at least one protein selected from the group consisting of said proteins (C) to (F) is increased; an L-proline-producing bacterium in which an expression amount of at least one protein selected from the group consisting of said proteins (A) to (F) is increased; an L-threonine-producing bacterium in which an expression amount of at least one protein selected from the group consisting of said proteins (E) and (F) is increased; an L-arginine-producing bacterium in which an expression amount of at least one protein selected from the group consisting of said proteins (G) and (H) is increased; or an L-isoleucine-producing bacterium in which an expression amount of at least one protein selected from the group consisting of said proteins (C) and (D) is increased.

[0010] Preferably, in the bacterium of the present invention, a copy number of a DNA coding for said protein in a cell is increased. The DNA is preferably carried on a multicopy vector in the cell or on a transposon in the cell.

[0011] The present invention also provides a method for producing an L-amino acid, comprising the steps of:

cultivating the bacterium of the present invention in a culture medium, to produce and accumulate the L-amino acid in the medium, and  
recovering the L-amino acid from the medium (hereinafter also referred to as "the bacterium of the present invention").

[0012] The method of the present invention preferably an L-lysine production method using an L-lysine-producing bacterium in which an expression amount of at least one protein selected from the group consisting of the proteins (A) to (D), (G) and (H) is increased; an L-glutamic acid production method using an L-glutamic acid-producing bacterium in which an expression amount of at least one protein selected from the group consisting of the proteins (A) to (H) is increased; an L-alanine production method using an L-alanine-producing bacterium in which an expression amount of at least one protein selected from the group consisting of the proteins (C) and (D) is increased; an L-valine production method using an L-valine-producing bacterium in which an expression amount of at least one protein selected from the group consisting of the proteins (C) and (D) is increased; an L-histidine production method using an L-histidine-producing bacterium in which an expression amount of at least one protein selected from the group consisting of said proteins (C) to (F) is increased; an L-proline production method using an L-proline-producing bacterium in which an expression amount of at least one protein selected from the group consisting of said proteins (A) to (F) is increased; an L-threonine production method using an L-threonine-producing bacterium in which an expression amount of at least one protein selected from the group consisting of said proteins (E) and (F) is increased; an L-arginine production method using an L-arginine-producing bacterium in which an expression amount of at least one protein selected from the group consisting of said proteins (G) and (H) is increased; or an L-isoleucine production method using an L-isoleucine-producing bacterium in which an expression amount of at least one protein selected from the group consisting of said proteins (C) and (D) is increased.

[0013] Preferably, in the method of the present invention, a copy number of a DNA coding for said protein in a cell of the bacterium is increased. The DNA is preferably carried on a multicopy vector in the cell, or on a transposon in the cell.

[0014] According to the present invention, an ability to produce an L-amino acid of a bacterium belonging to the genus *Escherichia* can be increased. Also, a method for producing an L-amino acid can be improved in a production rate of an L-amino acid.

[0015] The present invention will be explained in detail below. Hereinafter, an amino acid is of L-configuration unless otherwise noted.

#### (1) Bacterium of the present invention

[0016] The bacterium of the present invention is a bacterium belonging to the genus *Escherichia* and having an ability to produce an amino acid, in which the ability to produce the amino acid is increased by increasing an expression amount of a protein which has an activity of increasing the ability to produce the amino acid of the bacterium, or an activity of increasing resistance to an amino acid or amino acid analogue. Hereinafter, the protein is referred to as "amino acid excretion protein" for the sake of convenience. However, the term does not mean that function of the protein

is limited to amino acid excretion.

[0017] Examples of the amino acid excretion protein include a protein having an amino acid sequence shown in SEQ ID NO: 10, a protein having an amino acid sequence shown in SEQ ID NO: 12, a protein having an amino acid sequence shown in SEQ ID NO: 14 and a protein having an amino acid sequence shown in SEQ ID NO: 16.

[0018] The amino acid excretion protein may have selectivity to amino acid. An amino acid excretion protein appropriate for each amino acid can be determined by allowing the amino acid excretion protein to be expressed in a bacterium belonging to the genus *Escherichia* and having an ability to produce the amino acid, and measuring an increase of a yield of the amino acid or measuring an increase of a minimum inhibition concentration (MIC) of an amino acid or amino acid analogue.

[0019] For example, in the case of lysine, a protein having an amino acid sequence shown in SEQ ID NO: 10, 12 or 16 is effective; in the case of glutamic acid, a protein having an amino acid sequence shown in SEQ ID NO: 10, 12, 14 or 16 is effective; in the case of alanine, a protein having an amino acid sequence shown in SEQ ID NO: 12 is effective; in the case of valine, a protein having an amino acid sequence shown in SEQ ID NO: 12 is effective; in the case of histidine, a protein having an amino acid sequence shown in SEQ ID NO: 12 or 14; in the case of proline, a protein having an amino acid sequence shown in SEQ ID NO: 10, 12 or 14 is effective; in the case of threonine, a protein having an amino acid sequence shown in SEQ ID NO: 14 is effective; in the case of arginine, a protein having an amino acid sequence shown in SEQ ID NO: 16 is effective; and in the case of isoleucine, a protein having an amino acid sequence shown in SEQ ID NO: 12 is effective.

[0020] The term "an expression amount is increased" used herein usually means that the expression amount is larger than that in a wild strain of *E. coli* such as strain MG1655 or W3110. The term also means that when a strain is obtained by modification through genetic engineering techniques or the like, the expression amount is larger than that prior to the modification. The expression amount of the amino acid excretion protein may be determined directly by the determination of the amino acid excretion protein or indirectly by the determination of MIC of an amino acid or amino acid analogue or of amino acid productivity of a bacterium belonging to the genus *Escherichia* and having the amino acid excretion protein.

[0021] The method for increasing the expression amount of the amino acid excretion protein is exemplified by a method for increasing a copy number of DNA encoding the amino acid excretion protein in a cell of the bacterium.

[0022] For increasing the copy number in the cell, a DNA fragment coding for the amino acid excretion protein may be ligated to a vector which functions in a bacterium belonging to the genus *Escherichia* to produce a recombinant DNA, which is introduced to a host to transform it. The copy number of the gene coding for the amino acid excretion protein (amino acid excretion protein gene) in the cell of the transformant strain increases, thereby increasing the expression amount of the amino acid excretion protein. The vector is preferably a multicopy vector.

[0023] The increase of the copy number in the cell can be achieved by allowing plural copies of the amino acid excretion protein gene to exist on chromosomal DNA of the host. The introduction of plural copies of the amino acid excretion protein gene to chromosomal DNA of a bacterium belonging to the genus *Escherichia*, may be conducted through homologous recombination by using a sequence of which plural copies exist on the chromosomal DNA, as a target. As the sequence of which plural copies exist on the chromosomal DNA, a repetitive DNA and an inverted repeat present in a terminal portion of a transposable element may be used. Alternatively, as disclosed in Japanese Patent Application Laid-Open No. 2-109985 (1990), the plural copies can be introduced to the chromosomal DNA by making the amino acid excretion protein gene carried on a transposon and allowing the transposon to be transposed, which is preferred. According to any of the above-mentioned methods, the copy number of the amino acid excretion protein gene in the transformant strain increases, thereby increasing the expression amount of the amino acid excretion protein.

[0024] The multicopy vector is exemplified by plasmid vectors such as pBR322, pMW118, pUC19 or the like, and phage vectors such as  $\lambda$ 1059,  $\lambda$ BF101, M13mp9 or the like. The transposon is exemplified by Mu, Tn10, Tn5 or the like.

[0025] The introduction of a DNA into a bacterium belonging to the genus *Escherichia* can be performed, for example, by a method of D. M. Morrison (Methods in Enzymology 68, 326 (1979)) or a method in which recipient bacterial cells are treated with calcium chloride to increase permeability of DNA (Mandel, M. and Higa, A., J. Mol. Biol., 53, 159 (1970)) and the like.

[0026] Besides the above-mentioned gene amplification, the increase of the expression amount of the amino acid excretion protein can be also achieved by replacing an expression regulatory sequence such as a promoter of the amino acid excretion protein gene with stronger one (see Japanese Patent Application Laid-Open No. 1-215280 (1989)). For example, *lac* promoter, *trp* promoter, *tac* promoter,  $P_R$  promoter and  $P_L$  promoter of lambda phage, and the like are known as a strong promoter. The replacement with the promoter enhances expression of the amino acid excretion protein, thereby increasing the expression amount of the amino acid excretion protein. The enhancement of the expression regulatory sequence may be combined with the increase of the copy number of the amino acid excretion protein.

[0027] In the bacterium of the present invention, expression amounts of plural amino acid excretion proteins may be increased.

[0028] The amino acid excretion protein is encoded by genes which are known as *yahN* gene, *yeaS* gene, *yfiK* gene and *yggA* gene and of which functions are unknown. Therefore, the DNA encoding the amino acid excretion protein can be obtained by synthesizing primers based on the known sequences (for example, the entire nucleotide sequence of chromosome of *Escherichia coli* strain K-12 has been already determined (Science, 277, 1453-1474(1997))), and conducting amplification by PCR using chromosomal DNA of a bacterium belonging to the genus *Escherichia* as a template. Also, the object DNA fragment can be selected by hybridization from a chromosomal DNA library of a bacterium belonging to the genus *Escherichia* by preparing a probe based on the known sequences. Alternatively, the DNA encoding the amino acid excretion protein may be synthesized based on the known sequences. The nucleotide sequence of the DNA encoding the amino acid excretion protein is exemplified by that shown in SEQ ID NO: 9, 11, 13 or 15 in the Sequence Listing.

[0029] Methods for preparation of chromosomal DNA, preparation of chromosomal DNA library, hybridization, PCR, preparation of plasmid DNA, digestion and ligation of DNA, transformation, selection of an oligonucleotide as a primer and the like may be ordinary methods well known to one skilled in the art. These methods are described in Sambrook, J., Fritsch, E. F., and Maniatis, T., "Molecular Cloning A Laboratory Manual, Second Edition", Cold Spring Harbor Laboratory Press (1989) and the like.

[0030] The amino acid excretion protein may comprise substitution, deletion, insertion, addition or inversion of one or several amino acids at one or a plurality of positions, provided that the activity of increasing the ability to produce the amino acid of the bacterium belonging to the genus *Escherichia* and having the protein is not deteriorated. The term "several" may vary depending on a position in a steric structure of the protein and a kind of an amino acid residue. It is because some amino acids such as isoleucine and valine have high similarity to each other, and a difference between such the amino acids does not largely affect the steric structure of the protein.

[0031] The DNA which codes for the substantially same protein as the amino acid excretion protein as described above, may be obtained, for example, by modifying the nucleotide sequence, for example, by means of the site-directed mutagenesis method so that one or more amino acid residues at a specified site involve substitution, deletion, insertion, addition or inversion. The DNA modified as described above may be obtained by the conventionally known mutation treatment. The mutation treatment includes a method for treating a DNA coding for the amino acid excretion protein *in vitro*, for example, with hydroxylamine, and a method for treating a microorganism, for example, a bacterium belonging to the genus *Escherichia*, harboring a DNA coding for the amino acid excretion protein with ultraviolet irradiation or a mutating agent such as N-methyl-N'-nitro-N-nitrosoguanidine (NG) and nitrous acid usually used for the mutation treatment.

[0032] The substitution, deletion, insertion, addition or inversion of the one or more amino acid residues includes a naturally-occurring mutation or variation which is resulted from a difference between individual microorganisms having the amino acid excretion protein and a difference between species, strains or the like.

[0033] The DNA, which codes for substantially the same protein as the amino acid excretion protein, can be obtained by allowing a DNA having the mutation as described above to be expressed in a cell of an appropriate bacterium belonging to the genus *Escherichia*, and investigating the increase of amino acid productivity of the cell.

[0034] Also, the DNA, which codes for substantially the same protein as the amino acid excretion protein, can be obtained by isolating a DNA which hybridizes with DNA having, for example, a nucleotide sequence shown in SEQ ID NO: 9, 11, 13 or 15 in Sequence Listing under stringent conditions, and which codes for a protein having the activity of increasing the ability to produce the amino acid of the bacterium belonging to the genus *Escherichia*, from DNAs encoding the amino acid excretion proteins having mutations or cells containing the DNAs. The term "stringent conditions" referred to herein means a condition under which a specific hybrid is formed, and a non-specific hybrid is not formed. It is difficult to clearly express this condition by using any numerical value. However, for example, the stringent conditions include a condition under which DNAs having high homology, for example, DNAs having homology of not less than 70% with each other are hybridized, and DNAs having homology lower than the above with each other are not hybridized, or a condition of a salt concentration corresponding to 60°C, 1x SSC, 0.1% SDS, preferably 0.1x SSC, 0.1% SDS which is a washing condition of ordinary Southern hybridization.

[0035] Although there may be a gene in which a stop codon is made in the middle, or a gene encoding a protein losing the activity due to mutation of the active center among the genes which hybridize under such the condition, such genes can be easily eliminated by ligating the genes to a commercially available activity-expression vector and determining the activity of increasing the ability to produce the amino acid of the bacterium belonging to the genus *Escherichia* as described above.

[0036] The term "DNA coding for a protein" used herein means a DNA of which one of strands codes for the protein when the DNA is double-stranded.

[0037] By increasing an expression amount of an amino acid excretion protein in an amino acid-producing bacterium belonging to the genus *Escherichia* as described above, a produced amount of the amino acid can be increased. As the bacterium belonging to the genus *Escherichia* in which the expression amount of the amino acid excretion protein is to be increased, strains which have abilities to produce desired amino acids (amino acid productivities) are used.

Besides, an ability to produce an amino acid may be imparted to a bacterium in which the expression amount of the amino acid excretion protein is increased. Examples of amino acid-producing bacteria belonging to the genus *Escherichia* include *E. coli* AJ13199 (FR patent No. 2747689), and those obtainable from known materials (e.g., *E. coli* W3110 (tyrA)/pCABD2, *E. coli* VL614, *E. coli* VL2054, *E. coli* VL2160, *E. coli* VL2151, *E. coli* W3350 argE::Tn10/pKA10 as described in the Examples below).

[0038] For reference, the amino acid excretion protein according to the present invention was identified for the first time as described below.

[0039] The present inventors have identified *rhtB* and *rhtC* as threonine excretion protein genes of a bacterium belonging to the genus *Escherichia*. The present inventors searched databases based on a hypothesis that amino acid excretion proteins may share a common structure. Namely, BLAST and PSI-BLAST search (Altschul, S.F. et al., *Nucleic Acids Res.*, 25, 3389-3402(1997)) for homology of a protein encoded by *rhtB* was performed in GenBank CDS, PDB, SWISS-PROT, Spupdate and PIR. Tblastn search was performed in unfinished microbial genomes. BLITZ search (Sturrock, S.S., and Collins, J.F., Mpsch version 1.3. Biocomputing research unit University of Edinburgh, UK (1993)) was performed in SWALL database. SMART search (Ogiwara, I. et al., *Protein Sci.*, 5, 1991-1999 (1996)) was performed in the databases of translations and SWISS-PROT. From the samples of more than 60 sequences found, YeaS (corresponding to f212 of ACCESSION No. AE000274 in GenBank), YahN (corresponding to f223 of ACCESSION No. AE000140 in GenBank), YfiK (corresponding to o195 of ACCESSION No. AE000344 in GenBank) and YggA (corresponding to f211 of ACCESSION No. AE000375 in GenBank) remained as proteins which may have similar function to *RhtB*, among those originating from *E. coli*. Since functions of any of these genes were unknown, the genes were actually obtained, and effects thereof on MIC of amino acids and amino acid analogues and on amino acid production were examined by enhancing activities thereof. As a result, an effect of increasing MIC of some amino acids and analogues was found with respect to YeaS, YfiK, YahN and YggA. Further examination has revealed that proteins encoded by these genes exhibit an effect of increasing an amino acid accumulation, although they may have some amino acid selectivities.

## (2) Method of the present invention

[0040] The method of the present invention comprises the steps of cultivating the bacterium of the present invention, in a culture medium, to produce and accumulate the amino acid in the medium, and recovering the amino acid from the medium.

[0041] Suitable amino acids include lysine, glutamic acid, alanine, valine, homoserine, proline, and threonine.

[0042] In the method of present invention, the cultivation of the bacterium belonging to the genus *Escherichia*, the collection and purification of amino acid from the liquid medium may be performed in a manner similar to those of the conventional method for producing an amino acid by fermentation using a bacterium. A medium used in cultivation may be either a synthetic medium or a natural medium, so long as the medium includes a carbon and a nitrogen source and minerals and, if necessary, nutrients which the bacterium used requires for growth in appropriate amounts. The carbon source may include various carbohydrates such as glucose and sucrose, and various organic acids. Depending on assimilatory ability of the used bacterium, alcohol including ethanol and glycerol may be used. As the nitrogen source, ammonia, various ammonium salts such as ammonium sulfate, other nitrogen compounds such as amines, a natural nitrogen source such as peptone, soybean hydrolyte and digested fermentative microbe are used. As minerals, monopotassium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, calcium carbonate are used.

[0043] The cultivation is preferably culture under an aerobic condition such as a shaking culture, and an aeration and stirring culture. The temperature of culture is usually 20 to 40°C, preferably 30 to 38°C. The pH of the culture is usually between 5 and 9, preferably between 6.5 and 7.2. The pH of the culture can be adjusted with ammonia, calcium carbonate, various acids, various bases, and buffers. Usually, a 1 to 3-day cultivation leads to the accumulation of the target amino acid in the medium.

[0044] Recovering the amino acid can be performed by removing solids such as cells from the medium by centrifugation or membrane filtration after cultivation, and then collecting and purifying the target amino acid by ion exchange, concentration and crystalline fraction methods and the like.

## Best Mode for Carrying Out the Invention

[0045] The present invention will be more concretely explained below with reference to Examples.

Example 1. Preparation of the DNA fragments which code for amino acid excretion proteins.

[0046] The entire nucleotide sequence of chromosome of *E. coli* strain K-12 has been determined (Science, 277,

1453-1474, 1997). Based on the reported nucleotide sequence, primers were synthesized and the genes *yahN*, *yfiK*, *yeaS* and *yggA* were amplified by PCR.

(1). Chromosomal DNA of the *E. coli* strain MG1655 was used as a template.

[0047] The chromosomal DNA was prepared by an ordinary method (Sambrook, J., Fritsch E. F. and Maniatis T. (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.). In the PCR reaction, a standard condition described in "PCR protocols. Current methods and applications". (White, B.A., ed. Humana Press, Totowa, New Jersey, 1993) was used. The obtained PCR products were purified by an ordinary method and digested with restriction enzymes as described below.

[0048] The *yahN* gene was amplified by using the primers No.1 and No. 2.

Primer No.1: gtgtggaaccgacgccggat (a sequence complementary to a sequence of from 1885 base to 1904 base in a nucleotide sequence registered under ACCESSION No. AE000140 in GenBank; SEQ ID NO: 17), and

Primer No.2: tgtgtatggtacggggttcgag (a sequence of from 223 base to 245 base in the same; SEQ ID NO: 18).

[0049] The obtained PCR product after purification was digested with restriction enzymes *Pst*I and *Stu*I and ligated to vector pUC21 (Vieira, Messing, Gene, 100, 189-194, 1991) digested with the enzymes *Pst*I and *Eco*RV by using a ligation kit. Then, transformation of competent cells of *E. coli* TG1 (Sambrook, J., Fritsch E. F. and Maniatis T. (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.) with the product was conducted and the cells were spread on L medium (10 g/l Bacto trypton, 5 g/l Yeast extract, 5 g/l NaCl, 15 g/l agar, pH 7.0) containing 10 mg/ml IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside) and 40 mg/ml X-gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside) and 100 mg/ml ampicillin, and cultured overnight. Appeared white colonies were picked up and subjected to single colony isolation to obtain transformants. Plasmid was prepared from the transformants using an alkali extraction method and designated as pYAHN.

[0050] The *yeaS* gene was amplified by using the primers No.3 and No. 4.

Primer No.3: ctttgcaatcccgtctccc (a sequence complementary to a sequence of from 7683 base to 7702 base in a nucleotide sequence registered under ACCESSION No AE000274 in GenBank; SEQ ID NO: 19);

Primer No.4: gcccatgcataacggaaag (a sequence of from 5542 base to 5561 base in the same; SEQ ID NO: 19).

[0051] The obtained PCR product after purification was digested with a restriction enzyme *Ava*I and ligated to vector pUC19. After transformation of *E. coli* TG1 as above, the plasmid designated as pYEAS was obtained.

[0052] The *yfiK* gene was amplified by using the primers No.5 and No.6.

Primer No.5: gaagatctgtaggccgataaggcg (a sequence of from 4155 base to 4177 base in a nucleotide sequence registered under ACCESSION No AE000344 in GenBank, with a restriction enzyme *Bgl*II site added at the 5'-end thereof; SEQ ID NO: 21)

Primer No.6: tggttttaccaattggccgc (a sequence complementary to a sequence of from 6307 base to 6326 base in the same; SEQ ID NO: 22).

[0053] The obtained PCR product after purification was digested with restriction enzymes *Bgl*II and *Mun*I and ligated to vector pUC21 digested with restriction enzymes *Bgl*II and *Eco*RI. After transformation of *E. coli* TG1 as above, the plasmid designated pYFIK was obtained.

[0054] The *yggA* gene was amplified by using the primers No.7 and No.8.

Primer No.7: acttctcccgagccagttc (a sequence complementary to a sequence of from 9606 base to 9626 base in a nucleotide sequence registered under ACCESSION No AE000375 in GenBank; SEQ ID NO: 23).

Primer No.8: ggcaagcttagcgctctgtt (a sequence of from 8478 base to 8498 base in the same; SEQ ID NO: 24).

[0055] The obtained PCR product after purification was digested with restriction enzymes *Hind*III and *Cla*I and ligated to vector pOK12 (Vieira, Messing, Gene, 100, 189-194, 1991) digested with the same restriction enzymes. After transformation of *E. coli* TG1 as above, the plasmid designated pYGGA was obtained.



(2). Chromosomal DNA of the *E. coli* strain W3110 was used as a template.

[0056] The *yahN* gene was amplified by using the primers No.9 (SEQ ID NO 1) and No. 10 (SEQ ID NO.2)  
 [0057] The *yeaS* gene was amplified by using the primers No.11 (SEQ ID NO 3) and No.12 (SEQ ID NO 4)  
 [0058] The *yfiK* gene was amplified by using the primers No.13 (SEQ ID NO 5) and No.14 (SEQ ID NO 6).  
 [0059] The *yggA* gene was amplified by using the primers No.15 (SEQ ID NO 7) and No.16 (SEQ ID NO 8)  
 [0060] The obtained PCR product was purified, digested with restriction enzymes *SacI* and *XbaI* (*EcoRI* and *PstI* for *yggA*), and ligated to plasmid pMW118 (Nippon Gene). The plasmid into which a DNA fragment of which sequence was identical to the reported sequence was inserted was designated as follows:

One carrying *yahN*: pMW118::*yahN*  
 One carrying *yeaS*: pMW118::*yeaS*  
 One carrying *yfiK*: pMW118::*yfiK*  
 One carrying *yggA*: pMW118::*yggA*

**Example 2.** Effect of the *yahN*, *yeaS*, *yfiK*, and *yggA* DNA fragments amplification on the *E. coli* TG1 resistance to some amino acids and amino acid analogues.

[0061] The homology of the *yeaS*, *yfiK*, *yahN* and *yggA* gene products with the lysine transporter, LysE, of *Corynebacterium glutamicum* (Vrjic et al., Mol. Microbiol., 22, 815-826, 1996) and RhtB protein involved in homoserine excretion, indicates the analogues function for these proteins. It is well known that the increased expression of the genes involved in antibiotic and heavy metal efflux increases the level of resistance to the drugs (Nikaido, H. J. Bacteriology, 178, 5853-5859, 1996). Therefore, the effect of the pYEAS, pYAHN, pYFIK, and pYGGA plasmids on susceptibility of the strain TG1 to some amino acids and amino acid analogues was tested. Overnight cultures of the *E. coli* strains TG1/pYEAS, TG1/pYAHN, TG1/pYFIK, TG1/pYGGA and of the control strains TG1/pUC21, TG1/pUC19 and TG1/pOK12 grown in M9 minimal medium with an appropriate antibiotic on a rotary shaker ( $10^9$  cfu/ml) were diluted 1:100 in M9 minimal medium and grown for 5 h in the same medium. Then the log phase cultures thus obtained were diluted and about  $10^4$  alive cells were applied to well-dried test plates with M9 agar containing doubling increments of amino acids or analogues. Thus the minimum inhibition concentration (MIC) of these compounds were examined.

[0062] The results are shown in Table 1. It follows from the Table 1 that multiple copies of *yfiK* gene conferred increased resistance to proline, homoserine, histidine, threonine, glutamate, lysine,  $\alpha$ -amino- $\beta$ -hydroxyvaleric-acid (AHVA), S-(2-aminoethyl)-L-cysteine (AEC) and  $\alpha$ -aminobutyric acid; multiple copies of *yahN* gene conferred increased resistance to proline, multiple copies of *yeaS* gene conferred increased resistance to threonine, homoserine, lysine, glutamate, histidine, praline and  $\alpha$ -aminobutyric acid; multiple copies of *yggA* gene conferred increased resistance to S-(2-aminoethyl)-L-cysteine (AEC), lysine, and arginine. These results indicate that except for *YahN*, every of the presumed transporters have specificity to several substrates (amino acids and amino acid analogues), or may show non-specific effects as a result of amplification.

Table 1

Substrate	MIC ( $\mu$ g/ml) for <i>E. coli</i> TG1, harboring the plasmid				
	pUC21	pYFIK	pYAHN	pYEAS	pYGGA
L-homoserine	500	1000	500	1000	500
L-threonine	30000	40000	30000	50000	30000
L-lysine	5000	7500	5000	7500	15000
L-glutamate (Na salt)	5000	10000	5000	20000	5000
L-histidine	5000	10000	5000	30000	5000
L-valine	0.5	0.5	0.5	0.5	0.5
L-proline	1000	5000	2000	2000	1000
L-arginine	10000	10000	10000	10000	20000
AHVA	100	200	100	100	100
AEC	5	10	5	5	200



Table 1 (continued)

Substrate	MIC ( $\mu\text{g/ml}$ ) for <i>E. coli</i> TG1, harboring the plasmid				
	pUC21	pYFIK	pYAHN	pYEAS	pYGGA
$\alpha$ -aminobutyric acid	2500	5000	2500	>10000	2500
4-aza-DL-leucine	100	100	100	100	100

Example 3. Effect of *yeaS*, *yahN*, and *yfiK* DNA fragments amplification on glutamic acid production.

[0063] The *E. coli* strain AJ13199 (FR patent No. 2747689) was transformed with the vector pUC21 and each of the plasmids pYAHN, pYEAS and pYFIK. Thus the strains AJ13199/pUC21 (VKPM B-7728), AJ13199/pYAHN (VKPM B-7729), AJ13199/pYEAS (VKPM B-7731), and AJ13199/pYFIK (VKPM B-7730) were obtained.

[0064] These strains were each cultivated at 37°C for 18 hours in a nutrient broth with 100 mg/l ampicillin, and 0.3 ml of the obtained culture was inoculated into 3 ml of a fermentation medium containing 100 mg/l ampicillin, in a 20 x 200 mm test tube, and cultivated at 37°C for 48 hours with a rotary shaker. After the cultivation, an accumulated amount of glutamic acid in the medium was determined by known method.

[0065] The composition of the fermentation medium (g/l):

Glucose	80
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	22
K <sub>2</sub> HPO <sub>4</sub>	2
NaCl	0.8
MgSO <sub>4</sub> · 7H <sub>2</sub> O	0.8
FeSO <sub>4</sub> · 7H <sub>2</sub> O	0.02
MnSO <sub>4</sub> · 5H <sub>2</sub> O	0.02
Thiamine HCl	0.0002
Yeast extract	1.0
CaCO <sub>3</sub>	30.0 (dry-heat-sterilized at 180°C for 2 h)
(Glucose and K <sub>2</sub> HPO <sub>4</sub> separately sterilized)	

[0066] The results are shown in Table 2. As shown in Table 2, the strains AJ13199/pYAHN, AJ13199/pYEAS, and AJ13199/pYFIK accumulated glutamic acid in a larger amount than the strain AJ13199/pUC21 in which an expression amount of amino acid excretion proteins was not enhanced.

Table 2

Strain	Glutamic acid, g/l
AJ13199/pUC21	21.9
AJ13199/pYAHN	27.9
AJ13199/pYEAS	29.7
AJ13199/pYFIK	28.4

Example 4. Effect of *yeaS*, *yahN*, and *yfiK* DNA fragments amplification on lysine production.

[0067]

(1). As the lysine-producing bacterium belonging to the genus *Escherichia*, *E. coli* strain W3110 (TyrA) described

in European Patent Publication No. 488424 to which plasmid pCABD2 was introduced, described in International Publication No. WO 95/16042) was used. Specifically, plasmid pCABD2, and each of the plasmid pMW118::y<sub>ah</sub>N, pMW118::y<sub>ea</sub>S, pMW118::y<sub>fi</sub>K and pMW118 were introduced to *E. coli* strain W3110 (TyrA) to obtain the following strains:

W3110 (tyrA)/pCABD2+pMW118::y<sub>ah</sub>N  
 W3110 (tyrA)/pCABD2+pMW118::y<sub>ea</sub>S  
 W3110 (tyrA)/pCABD2+pMW118::y<sub>fi</sub>K  
 W3110 (tyrA)/pCABD2+pMW118.

Lysine productivity of these strains was estimated by culture. The composition of the used medium was as follows (g/l):

Glucose	40.0
MgSO <sub>4</sub> · 7H <sub>2</sub> O	1.0
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	16.0
K <sub>2</sub> HPO <sub>4</sub>	1.0
FeSO <sub>4</sub> · 7H <sub>2</sub> O	0.01
MnSO <sub>4</sub> · 7H <sub>2</sub> O	0.01
Yeast extract (Difco)	2.0
Tyrosine	0.1
Adjusted to pH 7.0 and autoclaved at 115° C for 10 minutes. (Glucose and MgSO <sub>4</sub> · 7H <sub>2</sub> O separately sterilized)	
Pharmacopeial CaCO <sub>3</sub> 25 g/l (dry-heat-sterilized at 180°C for 2 h)	

As antibiotics, 20 mg/l of streptomycin and 50 mg/l of ampicillin were added depending on a kind of a plasmid. Cultivation was conducted at 37°C for 30 hours with agitation at 115 rpm. The results are shown in Table 3.

Table 3

Strain	Lysine, g/l	Yield, (%)
W3110(tyrA)	0.08	0.2
W3110(tyrA)/pCABD2 + pMW118	12.2	30.5
W3110(tyrA)/pCABD2 + pMW118::y <sub>ah</sub> N	13.8	34.5
W3110(tyrA)/pCABD2 + pMW118::y <sub>ea</sub> S	12.7	31.8
W3110(tyrA)/pCABD2 + pMW118::y <sub>fi</sub> K	12.2	30.5

The result in Table 3 shows that the produced amount and the yield based on consumed sugar of lysine is increased by enhancement of Y<sub>ah</sub>N and Y<sub>ea</sub>S.

(2). As the lysine-producing bacterium belonging to the genus *Escherichia*, *E. coli* strain VL614 was used. This strain is a derivative of the known *E. coli* strain VL613 (SU Patent No. 1354458). In turn, the strain VL613 was obtained from the known strain Gif102 (Theze, J. and Saint Girons. J.Bacteriol., 118, 990-998, 1974) in the three steps:

At the first step the mutants resistant to 2 mg/ml S-(2-aminoethyl)-L-cysteine were selected and among them the strain VL611 was found capable to produce L-lysine.

At the second step the genes involved in sucrose utilization and located on the transposon Tn2555 (Doroshenko et al., Mol. Biologiya, 22, 645-658, 1988), were introduced into VL611 using phage P1-mediated trans-

duction giving the strain VL612.

At the third step, the mutation *rhtA23* from the strain VKPM B-3996, conferring resistance to threonine and homoserine (US Patent No. 5,175,107) was introduced into VL612 by phage P1 transduction giving the strain VL613.

[0068] The *E. coli* strain VL614 was obtained by transduction of the wild-type allele of the *rhtA* gene from the *E. coli* strain VKPM B-6204 (MG1655 *zbi3058::Tn10*) to VL613. Transductants were selected on L-medium containing 10 mg/l tetracyclin, and among them the strain VL614 (*rhtA*<sup>+</sup>) sensitive to 10 g/l homoserine was found.

[0069] The strain VL614 was transformed with the pYGGA plasmid or with the pOK12 vector to obtain strains VL614/pYGGA (VKPM B-7719) and VL614/pOK12 (VKPM B-7722).

[0070] These strains were each cultivated at 37°C for 18 hours in a nutrient broth with 50 mg/l kanamycin, and 0.3 ml of the obtained culture was inoculated into 3 ml of a fermentation medium (Example 3) containing 0.3 g/l threonine, 0.3 g/l methionine and 50 mg/l kanamycin, in a 20 x 200 mm test tube, and cultivated at 37°C for 48 hours with a rotary shaker. After the cultivation, each accumulated amount of lysine and glutamate in the medium was determined by the known method.

[0071] The results are shown in Table 4.

Table 4

Strain	Lysine, g/l	Glutamate, g/l
VL614/pOK12	2.6	0.8
VL614/pYGGA	3.6	2.2

[0072] As shown in Table 4, the strain VL614/pYGGA accumulated lysine in a larger amount than the strain VL614/pOK12 in which the *yggA* gene was not enhanced. Besides, the strain VL614/pYGGA accumulated more glutamic acid than the strain VL614/pOK12.

**Example 5.** Effect of *yeaS*, *yahN*, and *yfiK* DNA fragments amplification on threonine, alanine, valine and isoleucine production.

[0073] As the threonine-producing bacterium belonging to the genus *Escherichia*, the *E. coli* strain VL2054 was used. This strain was derived from the known *E. coli* strain VKPM B-3996 (US Patent No. 5,175,107) as follows.

[0074] Initially, a new recipient strain was constructed in several steps:

- The plasmidless derivative of the strain VKPM B-3996 was selected after spontaneous elimination of pVIC40 plasmid.
- The wild-type allele of the *rhtA* gene from the *E. coli* strain VKPM B-6204 (MG1655 *zbi3058::Tn10*) was introduced into the thus obtained strain by phage P1 mediated transduction as in the Example 4.
- A mutation inactivating *kan* gene of the Tn5 transposon inserted into the *tdh* gene was obtained after NG mutagenesis and selection of kanamycin-sensitive cells still unable to degrade threonine. Thus the strain VL2053 was obtained.

[0075] On the other hand, the threonine operon from pVIC40 was cloned into integrative Mud vector under the P<sub>R</sub> promoter of the phage lambda. In addition, the *cat* gene of Tn9 conferring the resistance to chloramphenicol was cloned into the same vector. The construct thus obtained was inserted into the chromosome of the *E. coli* strain C600 by use of the known method (US Patent No. 5,595,889) and transduced from the thus obtained strain to VL2053, giving the new plasmidless threonine-producing strain VL2054. This strain accumulated in culture broth also alanine, valine and isoleucine.

[0076] The strain VL2054 was transformed with each of the plasmids pYEAS, pYFIK, and with the vector pUC21 to obtain *E. coli* strains VL2054/pYEAS (VKPM B-7707), VL2054/pYFIK (VKPM B-7712) and VL2054/pUC21 (VKPM B-7708).

[0077] These strains were each cultivated at 37°C for 18 hours in a nutrient broth with 100 mg/l ampicillin, and 0.3 ml of the obtained culture was inoculated into 3 ml of a fermentation medium (Example 3) containing 100 mg/l ampicillin, in a 20 x 200 mm test tube, and cultivated at 37°C for 48 hours with a rotary shaker. After the cultivation, each accumulated amount of threonine, alanine, valine and isoleucine in the medium was determined by known method.

[0078] The results are shown in Table 5.

[0079] As shown in Table 5, the strain VL2054/pYFIK accumulated threonine in a larger amount than the strain VL2054/pUC21 in which the *yfiK* gene was not enhanced. Besides, the strain VL2054/pYEAS accumulated more alanine, valine and isoleucine than the strain VL2054/pUC21 in which the *yeaS* gene was not enhanced.

Table 5

Strain	Amino acid accumulation, g/l			
	Threonine	Alanine	Valine	Isoleucine
VL2054/pUC21	5.8	0.4	0.31	0.15
VL2054/pYEAS	5.2	1.4	0.52	0.45
VL2054/pYFIK	8.8	0.5	0.22	0.14

**Example 6.** Effect of *yeaS* and *yfiK* DNA fragments amplification on histidine production.

[0080] As the histidine-producing bacterium belonging to the genus *Escherichia*, the strains *E. coli* VL2160 was used. This strain was obtained on the basis of the known strain NK5526 *hisG*::Tn10 (VKPM B-3384) by phage P1-mediated transduction of the *hisG*<sup>R</sup> mutation desensitizing ATP-phosphoribosyltransferase from the strain CC46 (Astvatsaturianz et al., Genetika, 24, 1928-1934, 1988). The strain *E. coli* VL2160 was transformed with each of the plasmids pYEAS, pYFIK, and with the vectors pUC21 to obtain *E. coli* strains VL2160/pYEAS (VKPM B-7753), *E. coli* VL2160/pYFIK (VKPM B-7754), *E. coli* VL2160/pUC21 (VKPM B-7752).

[0081] These strains were each cultivated at 37°C for 18 hours in a nutrient broth with 100 mg/l ampicillin, and 0.3 ml of the obtained culture was inoculated into 3 ml of the fermentation medium (Example 3) containing an increased amount of yeast extract (3 g/l) and 100 mg/l ampicillin, in a 20 x 200 mm test tube, and cultivated at 34°C for 68 hours with a rotary shaker.

[0082] After the cultivation, an accumulated amount of histidine in the medium was determined by known method. The results are shown in Table 6.

Table 6

Strain	Histidine, g/l
VL2160/pUC21	1.2
VL2160/pYEAS	1.8
VL2160/pYFIK	1.4

[0083] As shown in Table 6, the strains *E. coli* VL2160/pYEAS and *E. coli* VL2160/pYFIK accumulated histidine in a larger amount than the strain *E. coli* VL2160/pUC21 in which the *yeaS* and *yfiK* genes were not enhanced.

**Example 7.** Effect of *yahN*, *yfiK* and *yeaS* DNA fragments amplification on proline production.

[0084] As the proline-producing bacterium belonging to the genus *Escherichia*, the strain VL2151 (W3350 *proB*<sup>+</sup>  $\Delta$ *putAP* Tn10) was used. This strain was obtained by transduction into W3350 of  $\Delta$ *putAP* mutation linked to Tn10 and selecting tetracycline-resistant transductants unable to utilize proline as a sole carbon source. The thus obtained strain W3350  $\Delta$ *putAP* Tn10 was mutagenized with NG and mutants resistant to 20 mg/l of 3,4-dehydro-DL-proline were selected. Among them the strain VL2151 (W3350 *proB*<sup>+</sup>  $\Delta$ *putAP* Tn10) was found capable to produce proline.

[0085] The strain *E. coli* VL2151 was transformed with each of the plasmids pYEAS, pYFIK, pYAHN and with the vectors pUC21 to obtain *E. coli* strains VL2151/pYEAS (VKPM B-7714), VL2151/pYFIK (VKPM B-7713), VL2151/pYAHN (VKPM B-7748) and *E. coli* VL2151/pUC21 (VKPM B-7715).

[0086] These strains were each cultivated at 37°C for 18 hours in a nutrient broth with 100 mg/l ampicillin, and 0.3 ml of the obtained culture was inoculated into 3 ml of a fermentation medium (Example 3) containing 100 mg/l ampicillin, in a 20 x 200 mm test tube, and cultivated at 37°C for 48 hours with a rotary shaker. After the cultivation, an accumulated amount of proline in the medium was determined by known method. The results are shown in Table 7.

Table 7

Strain	Proline, g/l
VL2151/pUC21	1.8
VL2151/pYAHN	2.2
VL2151/pYEAS	2.1
VL2151/pYFIK	2.5

[0087] As shown in Table 7, the strains *E. coli* VL2151/pYFIK, *E. coli* VL2151/pYAHN and *E. coli* VL2151/pYEAS accumulated proline in a larger amount than the strain *E. coli* VL2151/pUC21 in which the *yfiK*, *yahN* and *yeaS* genes were not enhanced. The amplification of *yfiK* gene had the most pronounced effect.

**Example 8.** Effect of *yggA* DNA fragments amplification on arginine production.

[0088] As arginine-producing bacterium belonging to the genus *Escherichia*, the strain W3350 *argE*::Tn10/pKA10 was used. This strain harbors a plasmid, pKA10, containing DNA region from *Corynebacterium (Brevibacterium) flavum* which complements at least *argA* and *argE* mutations in the recipient strain of *E. coli* K-12 (Kharitonov A. and Tarasov A.P. Molecular Genetics, Microbiology and Virology. No.9, 29-33, 1986).

[0089] The strain *E. coli* W3350 *argE*::Tn10/pKA10 was transformed with the plasmid pYGGA, or with the vector pOK12 to obtain the strains *E. coli* W3350 *argE*::Tn10/pKA10, pYGGA (VKPM B-7716) and *E. coli* W3350 *argE*::Tn10/pKA10, pOK12 (VKPM B-7718).

[0090] The thus obtained transformants were each cultivated at 37°C for 18 hours in a nutrient broth with 100 mg/l ampicillin and 50 mg/l kanamycin, and 0.3 ml of the obtained culture was inoculated into 3 ml of a fermentation medium (Example 3) containing 100 mg/l ampicillin and 50 mg/l kanamycin, in a 20 x 200 mm test tube, and cultivated at 37°C for 48 hours with a rotary shaker. After the cultivation, an accumulated amount of arginine in the medium was determined by known method.

[0091] The results are shown in Table 8.

Table 8

Strain	Arginine, g/l
W3350 <i>argE</i> ::Tn10/pKA10, pOK12	0.11
W3350 <i>argE</i> ::Tn10/pKA10, pYGGA	0.46

[0092] As shown in Table 8, the strains *E. coli* W3350 *argE*::Tn10/pKA10, pYGGA accumulated arginine in a larger amount than the strain *E. coli* W3350 *argE*::Tn10/pKA10, pUC21 in which the *yggA* gene was not enhanced.

[0093] The following *E. coli* strains have been deposited (according to international deposition based on Budapest Treaty) in the Russian National Collection of Industrial Microorganisms (VKPM) on December 29, 1998 under the accession numbers shown in parenthesis.

AJ13199/pUC21 (VKPM B-7728)  
 AJ13199/pYAHN (VKPM B-7729)  
 AJ13199/pYEAS (VKPM B-7731)  
 AJ13199/pYFIK (VKPM B-7730)  
 VL614/pYGGA (VKPM B-7719)  
 VL614/pOK12 (VKPM B-7722)  
 VL2054/pYEAS (VKPM B-7707)  
 VL2054/pYFIK (VKPM B-7712)  
 VL2054/pUC21 (VKPM B-7708)  
 VL2160/pYEAS (VKPM B-7753)  
 VL2160/pYFIK (VKPM B-7754)  
 VL2160/pUC21 (VKPM B-7752)

EP 1 016 710 A2

VL2151/pYFIK (VKPM B-7713)

VL2151/pYEAS (VKPM B-7714)

VL2151/pYAHN (VKPM B-7748)

VL2151/pUC21 (VKPM B-7715)

5 W3350 *argE*::Tn10/pKA10, pYGGA (VKPM B-7716)

W3350 *argE*::Tn10/pKA10, pOK12 (VKPM B-7718)

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SEQUENCE LISTING

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	115	120	125	
25	cgc cgc gga tta att acc gat ctc tct aac ccg caa acc gtt tta ttt	432		
	Arg Arg Gly Leu Ile Thr Asp Leu Ser Asn Pro Gln Thr Val Leu Phe			
	130	135	140	
30	ttt atc agt att ttc tca gta aca tta aat gcc gaa aca cca aca tgg	480		
	Phe Ile Ser Ile Phe Ser Val Thr Leu Asn Ala Glu Thr Pro Thr Trp			
	145	150	155	160
	gca cgt tta atg gcc tgg gcg ggg att gtg ctc gca tca att atc tgg	528		
	Ala Arg Leu Met Ala Trp Ala Gly Ile Val Leu Ala Ser Ile Ile Trp			
	165	170	175	
35	cga gtt ttt ctt agt cag gcg ttt tct ttg ccc gct gtg cgt cgt gct	576		
	Arg Val Phe Leu Ser Gln Ala Phe Ser Leu Pro Ala Val Arg Arg Ala			
	180	185	190	
40	tat ggg cgt atg caa cgc gtt gcc agt cgg gtt att ggt gca att att	624		
	Tyr Gly Arg Met Gln Arg Val Ala Ser Arg Val Ile Gly Ala Ile Ile			
	195	200	205	
45	ggt gta ttc gcg cta cgc ctg att tac gaa ggg gtg acg cag cgg tga	672		
	Gly Val Phe Ala Leu Arg Leu Ile Tyr Glu Gly Val Thr Gln Arg			
	210	215	220	
	<210> 10			
50	<211> 223			
	<212> PRT			
	<213> Escherichia coli			

&lt;400&gt; 10

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5      1                      5                      10                      15  
 Met Met Gln Leu Val His Leu Phe Met Asp Glu Ile Thr Met Asp Pro  
 Leu His Ala Val Tyr Leu Thr Val Gly Leu Phe Val Ile Thr Phe Phe  
                     20                      25                      30  
 Asn Pro Gly Ala Asn Leu Phe Val Val Val Gln Thr Ser Leu Ala Ser  
 10                      35                      40                      45  
 Gly Arg Arg Ala Gly Val Leu Thr Gly Leu Gly Val Ala Leu Gly Asp  
                     50                      55                      60  
 Ala Phe Tyr Ser Gly Leu Gly Leu Phe Gly Leu Ala Thr Leu Ile Thr  
 15                      65                      70                      75                      80  
 Gln Cys Glu Glu Ile Phe Ser Leu Ile Arg Ile Val Gly Gly Ala Tyr  
                     85                      90                      95  
 Leu Leu Trp Phe Ala Trp Cys Ser Met Arg Arg Gln Ser Thr Pro Gln  
 20                      100                      105                      110  
 Met Ser Thr Leu Gln Gln Pro Ile Ser Ala Pro Trp Tyr Val Phe Phe  
                     115                      120                      125  
 Arg Arg Gly Leu Ile Thr Asp Leu Ser Asn Pro Gln Thr Val Leu Phe  
                     130                      135                      140  
 25                      145                      150                      155                      160  
 Phe Ile Ser Ile Phe Ser Val Thr Leu Asn Ala Glu Thr Pro Thr Trp  
 Ala Arg Leu Met Ala Trp Ala Gly Ile Val Leu Ala Ser Ile Ile Trp  
                     165                      170                      175  
 30                      180                      185                      190  
 Arg Val Phe Leu Ser Gln Ala Phe Ser Leu Pro Ala Val Arg Arg Ala  
 Tyr Gly Arg Met Gln Arg Val Ala Ser Arg Val Ile Gly Ala Ile Ile  
                     195                      200                      205  
 35                      210                      215                      220  
 Gly Val Phe Ala Leu Arg Leu Ile Tyr Glu Gly Val Thr Gln Arg

&lt;210&gt; 11

&lt;211&gt; 639

&lt;212&gt; DNA

&lt;213&gt; Escherichia coli

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1)..(639)

&lt;400&gt; 11

gtg ttc gct gaa tac ggg gtt ctg aat tac tgg acc tat ctg gtt ggg 48

	Met	Phe	Ala	Glu	Tyr	Gly	Val	Leu	Asn	Tyr	Trp	Thr	Tyr	Leu	Val	Gly	
	1					5					10					15	
5	gcc	att	ttt	att	gtg	ttg	gtg	cca	ggg	cca	aat	acc	ctg	ttt	gta	ctc	96
	Ala	Ile	Phe	Ile	Val	Leu	Val	Pro	Gly	Pro	Asn	Thr	Leu	Phe	Val	Leu	
				20					25					30			
10	aaa	aat	agc	gtc	agt	agc	ggg	atg	aaa	ggc	ggg	tat	ctt	gcg	gcc	tgc	144
	Lys	Asn	Ser	Val	Ser	Ser	Gly	Met	Lys	Gly	Gly	Tyr	Leu	Ala	Ala	Cys	
				35				40					45				
15	ggg	gta	ttt	att	ggc	gat	gcg	gta	ttg	atg	ttt	ctg	gca	tgg	gct	gga	192
	Gly	Val	Phe	Ile	Gly	Asp	Ala	Val	Leu	Met	Phe	Leu	Ala	Trp	Ala	Gly	
		50					55					60					
20	gtg	gcg	aca	tta	att	aag	acc	acc	ccg	ata	tta	ttc	aac	att	gta	cgt	240
	Val	Ala	Thr	Leu	Ile	Lys	Thr	Thr	Pro	Ile	Leu	Phe	Asn	Ile	Val	Arg	
		65				70				75					80		
25	tat	ctt	ggg	gcg	ttt	tat	ttg	ctc	tat	ctg	ggg	agt	aaa	att	ctt	tac	288
	Tyr	Leu	Gly	Ala	Phe	Tyr	Leu	Leu	Tyr	Leu	Gly	Ser	Lys	Ile	Leu	Tyr	
					85				90					95			
30	gcg	acc	ctg	aag	ggg	aaa	aat	agc	gag	gcc	aaa	tcc	gat	gag	ccc	caa	336
	Ala	Thr	Leu	Lys	Gly	Lys	Asn	Ser	Glu	Ala	Lys	Ser	Asp	Glu	Pro	Gln	
				100				105					110				
35	tac	ggg	gct	att	ttt	aaa	cgc	gcg	tta	att	ttg	agc	ctg	act	aat	ccg	384
	Tyr	Gly	Ala	Ile	Phe	Lys	Arg	Ala	Leu	Ile	Leu	Ser	Leu	Thr	Asn	Pro	
				115				120					125				
40	aaa	gcc	att	ttg	ttc	tat	gtg	tcg	ttt	ttc	gta	cag	ttt	atc	gat	gtt	432
	Lys	Ala	Ile	Leu	Phe	Tyr	Val	Ser	Phe	Phe	Val	Gln	Phe	Ile	Asp	Val	
				130				135				140					
45	aat	gcc	cca	cat	acg	gga	att	tca	ttc	ttt	att	ctg	gcg	gcg	acg	ctg	480
	Asn	Ala	Pro	His	Thr	Gly	Ile	Ser	Phe	Phe	Ile	Leu	Ala	Ala	Thr	Leu	
		145				150				155				160			
50	gaa	ctg	gtg	agt	ttc	tgc	tat	ttg	agc	ttc	ctg	att	ata	tct	ggg	gct	528
	Glu	Leu	Val	Ser	Phe	Cys	Tyr	Leu	Ser	Phe	Leu	Ile	Ile	Ser	Gly	Ala	
				165				170				175					
55	ttt	gtc	acg	cag	tac	ata	cgt	acc	aaa	aag	aaa	ctg	gct	aaa	gtt	ggc	576
	Phe	Val	Thr	Gln	Tyr	Ile	Arg	Thr	Lys	Lys	Lys	Leu	Ala	Lys	Val	Gly	
				180				185				190					
60	aac	tca	ctg	att	ggg	ttg	atg	ttc	gtg	ggg	ttc	gct	gcc	cga	ctg	gcg	624
	Asn	Ser	Leu	Ile	Gly	Leu	Met	Phe	Val	Gly	Phe	Ala	Ala	Arg	Leu	Ala	
				195				200				205					
65	acg	ctg	caa	tcc	tga												639
	Thr	Leu	Gln	Ser													
				210													

&lt;210&gt; 12

&lt;211&gt; 212

&lt;212&gt; PRT

&lt;213&gt; Escherichia coli

&lt;400&gt; 12

Met Phe Ala Glu Tyr Gly Val Leu Asn Tyr Trp Thr Tyr Leu Val Gly  
 1 5 10 15  
 Ala Ile Phe Ile Val Leu Val Pro Gly Pro Asn Thr Leu Phe Val Leu  
 20 25 30  
 Lys Asn Ser Val Ser Ser Gly Met Lys Gly Gly Tyr Leu Ala Ala Cys  
 35 40 45  
 Gly Val Phe Ile Gly Asp Ala Val Leu Met Phe Leu Ala Trp Ala Gly  
 50 55 60  
 Val Ala Thr Leu Ile Lys Thr Thr Pro Ile Leu Phe Asn Ile Val Arg  
 65 70 75 80  
 Tyr Leu Gly Ala Phe Tyr Leu Leu Tyr Leu Gly Ser Lys Ile Leu Tyr  
 85 90 95  
 Ala Thr Leu Lys Gly Lys Asn Ser Glu Ala Lys Ser Asp Glu Pro Gln  
 100 105 110  
 Tyr Gly Ala Ile Phe Lys Arg Ala Leu Ile Leu Ser Leu Thr Asn Pro  
 115 120 125  
 Lys Ala Ile Leu Phe Tyr Val Ser Phe Phe Val Gln Phe Ile Asp Val  
 130 135 140  
 Asn Ala Pro His Thr Gly Ile Ser Phe Phe Ile Leu Ala Ala Thr Leu  
 145 150 155 160  
 Glu Leu Val Ser Phe Cys Tyr Leu Ser Phe Leu Ile Ile Ser Gly Ala  
 165 170 175  
 Phe Val Thr Gln Tyr Ile Arg Thr Lys Lys Lys Leu Ala Lys Val Gly  
 180 185 190  
 Asn Ser Leu Ile Gly Leu Met Phe Val Gly Phe Ala Ala Arg Leu Ala  
 195 200 205  
 Thr Leu Gln Ser  
 210

&lt;210&gt; 13

&lt;211&gt; 588

&lt;212&gt; DNA

&lt;213&gt; Escherichia coli

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1)..(588)

5 <400> 13  
 gtg aca ccg acc ctt tta agt gct ttt tgg act tac acc ctg att acc 48  
 Met Thr Pro Thr Leu Leu Ser Ala Phe Trp Thr Tyr Thr Leu Ile Thr  
 1 5 10 15

10 gct atg acg cca gga ccg aac aat att ctc gcc ctt agc tct gct acg 96  
 Ala Met Thr Pro Gly Pro Asn Asn Ile Leu Ala Leu Ser Ser Ala Thr  
 20 25 30

15 tcg cat gga ttt cgt caa agt acc cgc gtg ctg gca ggg atg agt ctg 144  
 Ser His Gly Phe Arg Gln Ser Thr Arg Val Leu Ala Gly Met Ser Leu  
 35 40 45

20 gga ttt ttg att gtg atg tta ctg tgt gcg ggc att tca ttt tca ctg 192  
 Gly Phe Leu Ile Val Met Leu Leu Cys Ala Gly Ile Ser Phe Ser Leu  
 50 55 60

25 gca gtg att gac ccg gca gcg gta cac ctt ttg agt tgg gcg ggg gcg 240  
 Ala Val Ile Asp Pro Ala Ala Val His Leu Leu Ser Trp Ala Gly Ala  
 65 70 75 80

30 gca tat att gtc tgg ctg gcg tgg aaa atc gcc acc agc cca aca aag 288  
 Ala Tyr Ile Val Trp Leu Ala Trp Lys Ile Ala Thr Ser Pro Thr Lys  
 85 90 95

35 gaa gac gga ctt cag gca aaa cca atc agc ttt tgg gcc agc ttt gct 336  
 Glu Asp Gly Leu Gln Ala Lys Pro Ile Ser Phe Trp Ala Ser Phe Ala  
 100 105 110

40 ttg cag ttt gtg aac gtc aaa atc att ttg tac ggt gtt acg gca ctg 384  
 Leu Gln Phe Val Asn Val Lys Ile Ile Leu Tyr Gly Val Thr Ala Leu  
 115 120 125

45 tcg acg ttt gtt ctg ccg caa aca cag gcg tta agc tgg gta gtt ggc 432  
 Ser Thr Phe Val Leu Pro Gln Thr Gln Ala Leu Ser Trp Val Val Gly  
 130 135 140

50 gtc agc gtt ttg ctg gcg atg att ggg acg ttt ggc aat gtg tgc tgg 480  
 Val Ser Val Leu Leu Ala Met Ile Gly Thr Phe Gly Asn Val Cys Trp  
 145 150 155 160

55 gcg ctg gcg ggg cat ctg ttt cag cga ttg ttt cgc cag tat ggt cgc 528  
 Ala Leu Ala Gly His Leu Phe Gln Arg Leu Phe Arg Gln Tyr Gly Arg  
 165 170 175

cag tta aat atc gtg ctt gcc ctg ttg ctg gtc tat tgc gcg gta cgc 576  
 Gln Leu Asn Ile Val Leu Ala Leu Leu Leu Val Tyr Cys Ala Val Arg  
 180 185 190

att ttc tat taa 588  
 Ile Phe Tyr  
 195



&lt;210&gt; 14

&lt;211&gt; 195

&lt;212&gt; PRT

&lt;213&gt; Escherichia coli

&lt;400&gt; 14

5

Met Thr Pro Thr Leu Leu Ser Ala Phe Trp Thr Tyr Thr Leu Ile Thr  
 1 5 10 15

Ala Met Thr Pro Gly Pro Asn Asn Ile Leu Ala Leu Ser Ser Ala Thr  
 20 25 30

15

Ser His Gly Phe Arg Gln Ser Thr Arg Val Leu Ala Gly Met Ser Leu  
 35 40 45

Gly Phe Leu Ile Val Met Leu Leu Cys Ala Gly Ile Ser Phe Ser Leu  
 50 55 60

20

Ala Val Ile Asp Pro Ala Ala Val His Leu Leu Ser Trp Ala Gly Ala  
 65 70 75 80

Ala Tyr Ile Val Trp Leu Ala Trp Lys Ile Ala Thr Ser Pro Thr Lys  
 85 90 95

25

Glu Asp Gly Leu Gln Ala Lys Pro Ile Ser Phe Trp Ala Ser Phe Ala  
 100 105 110

Leu Gln Phe Val Asn Val Lys Ile Ile Leu Tyr Gly Val Thr Ala Leu  
 115 120 125

30

Ser Thr Phe Val Leu Pro Gln Thr Gln Ala Leu Ser Trp Val Val Gly  
 130 135 140

Val Ser Val Leu Leu Ala Met Ile Gly Thr Phe Gly Asn Val Cys Trp  
 145 150 155 160

35

Ala Leu Ala Gly His Leu Phe Gln Arg Leu Phe Arg Gln Tyr Gly Arg  
 165 170 175

Gln Leu Asn Ile Val Leu Ala Leu Leu Leu Val Tyr Cys Ala Val Arg  
 180 185 190

40

Ile Phe Tyr  
 195

&lt;210&gt; 15

&lt;211&gt; 636

&lt;212&gt; DNA

&lt;213&gt; Escherichia coli

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1)..(636)

55

&lt;400&gt; 15

5	gtg ttt tct tat tac ttt caa ggt ctt gca ctt ggg gcg gct atg atc Met Phe Ser Tyr Tyr Phe Gln Gly Leu Ala Leu Gly Ala Ala Met Ile 1 5 10 15	48
10	cta ccg ctc ggt cca caa aat gct ttt gtg atg aat cag ggc ata cgt Leu Pro Leu Gly Pro Gln Asn Ala Phe Val Met Asn Gln Gly Ile Arg 20 25 30	96
15	cgt cag tac cac att atg att gcc tta ctt tgt gct atc agc gat ttg Arg Gln Tyr His Ile Met Ile Ala Leu Leu Cys Ala Ile Ser Asp Leu 35 40 45	144
20	gtc ctg att tgc gcc ggg att ttt ggt ggc agc gcg tta ttg atg cag Val Leu Ile Cys Ala Gly Ile Phe Gly Gly Ser Ala Leu Leu Met Gln 50 55 60	192
25	tcg ccg tgg ttg ctg gcg ctg gtc acc tgg ggc ggc gta gcc ttc ttg Ser Pro Trp Leu Leu Ala Leu Val Thr Trp Gly Gly Val Ala Phe Leu 65 70 75 80	240
30	ctg tgg tat ggt ttt ggc gct ttt aaa aca gca atg agc agt aat att Leu Trp Tyr Gly Phe Gly Ala Phe Lys Thr Ala Met Ser Ser Asn Ile 85 90 95	288
35	gag tta gcc agc gcc gaa gtc atg aag caa ggc aga tgg aaa att atc Glu Leu Ala Ser Ala Glu Val Met Lys Gln Gly Arg Trp Lys Ile Ile 100 105 110	336
40	gcc acc atg ttg gca gtg acc tgg ctg aat ccg cat gtt tac ctg gat Ala Thr Met Leu Ala Val Thr Trp Leu Asn Pro His Val Tyr Leu Asp 115 120 125	384
45	act ttt gtt gta ctg ggc agc ctt ggc ggg caa ctt gat gtg gaa cca Thr Phe Val Val Leu Gly Ser Leu Gly Gly Gln Leu Asp Val Glu Pro 130 135 140	432
50	aaa cgc tgg ttt gca ctc ggg aca att agc gcc tct ttc ctg tgg ttc Lys Arg Trp Phe Ala Leu Gly Thr Ile Ser Ala Ser Phe Leu Trp Phe 145 150 155 160	480
55	ttt ggt ctg gct ctt ctc gca gcc tgg ctg gca ccg cgt ctg cgc acg Phe Gly Leu Ala Leu Leu Ala Ala Trp Leu Ala Pro Arg Leu Arg Thr 165 170 175	528
60	gca aaa gca cag cgc att atc aat ctg gtt gtg gga tgt gtt atg tgg Ala Lys Ala Gln Arg Ile Ile Asn Leu Val Val Gly Cys Val Met Trp 180 185 190	576
65	ttt att gcc ttg cag ctg gcg aga gac ggt att gct cat gca caa gcc Phe Ile Ala Leu Gln Leu Ala Arg Asp Gly Ile Ala His Ala Gln Ala 195 200 205	624
70	ttg ttc agt tag	636

Leu Phe Ser

210

5

&lt;210&gt; 16

&lt;211&gt; 211

&lt;212&gt; PRT

10

&lt;213&gt; Escherichia coli

&lt;400&gt; 16

Met Phe Ser Tyr Tyr Phe Gln Gly Leu Ala Leu Gly Ala Ala Met Ile

15

1 5 10 15  
Leu Pro Leu Gly Pro Gln Asn Ala Phe Val Met Asn Gln Gly Ile Arg20 25 30  
Arg Gln Tyr His Ile Met Ile Ala Leu Leu Cys Ala Ile Ser Asp Leu

20

35 40 45  
Val Leu Ile Cys Ala Gly Ile Phe Gly Gly Ser Ala Leu Leu Met Gln50 55 60  
Ser Pro Trp Leu Leu Ala Leu Val Thr Trp Gly Gly Val Ala Phe Leu

25

65 70 75 80  
Leu Trp Tyr Gly Phe Gly Ala Phe Lys Thr Ala Met Ser Ser Asn Ile85 90 95  
Glu Leu Ala Ser Ala Glu Val Met Lys Gln Gly Arg Trp Lys Ile Ile

30

100 105 110  
Ala Thr Met Leu Ala Val Thr Trp Leu Asn Pro His Val Tyr Leu Asp115 120 125  
Thr Phe Val Val Leu Gly Ser Leu Gly Gly Gln Leu Asp Val Glu Pro

35

130 135 140  
Lys Arg Trp Phe Ala Leu Gly Thr Ile Ser Ala Ser Phe Leu Trp Phe145 150 155 160  
Phe Gly Leu Ala Leu Leu Ala Ala Trp Leu Ala Pro Arg Leu Arg Thr165 170 175  
Ala Lys Ala Gln Arg Ile Ile Asn Leu Val Val Gly Cys Val Met Trp

40

180 185 190  
Phe Ile Ala Leu Gln Leu Ala Arg Asp Gly Ile Ala His Ala Gln Ala

195 200 205

45

Leu Phe Ser

210

&lt;210&gt; 17

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

50

55

<220>

<223> Description of Artificial Sequence: primer for amplifying Escherichia coli yahN gene

<400> 17

gtgtggaacc gacgccgat

20

<210> 18

<211> 23

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer for amplifying Escherichia coli yahN gene

<400> 18

tggtgtatgg tacgggggttc gag

23

<210> 19

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer for amplifying Escherichia coli yeaS gene

<400> 19

ctttgccaat cccgtctccc

20

<210> 20

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer for amplifying Escherichia coli yeaS gene

<400> 20

gccccatgca taacggaaag

20

5 <210> 21  
<211> 26  
<212> DNA  
<213> Artificial Sequence

10 <220>  
<223> Description of Artificial Sequence: primer for amplifying Escherichia coli yfiK gene

15 <400> 21  
gaagatcttg taggccgat aaggcg 26

20 <210> 22  
<211> 20  
<212> DNA  
<213> Artificial Sequence

25 <220>  
<223> Description of Artificial Sequence: primer for amplifying Escherichia coli yfiK gene

30 <400> 22  
tggttttacc aattggccgc 20

35 <210> 23  
<211> 21  
<212> DNA  
<213> Artificial Sequence

40 <220>  
<223> Description of Artificial Sequence: primer for amplifying Escherichia coli yggA gene

45 <400> 23  
acttctcccg cgagccagtt c 21

50 <210> 24  
<211> 21  
<212> DNA  
<213> Artificial Sequence

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